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Published*With international search report.**Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.**With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.*(54) Title: **RECOVERING METALS FROM SOIL**

(57) Abstract

The invention relates to recovering metals, such as nickel and cobalt, by phytomining or phytoextracting soils rich in metals wherein the desired metal is selectively accumulated in hyperaccumulator plants by adjusting the soil pH. The metals are ultimately recovered from above-ground plant tissues at economically acceptable levels without further contaminating the metal-containing sites. The invention also relates to metal-hyperaccumulating plants.

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Recovering Metals from Soil

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

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Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Field of the Invention

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This invention pertains to methods for recovering metals, such as nickel and cobalt, from metal-rich soil using phytoextracting or phytomining techniques. Metals can be selectively extracted from soil by cultivating certain metal hyperaccumulating plants, such as *Alyssum* plants, on soil treated to adjust the pH.

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Related Art

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Industrial practices such as mining, smelting and disposing of manufacturing wastes have increased the concentrations of toxic metals in the environment. For example, at many nickel mining and smelting sites, levels of nickel and cobalt in soil have become so high that few plants survive; resulting in severe disruption of local ecosystems. Once nickel and cobalt enter soil, their removal is difficult since they are relatively immobile and they do not degrade into less toxic substances. The size of the areas affected by smelter and mine wastes are usually so large that engineering methods of soil remediation, such as soil removal and replacement, are too expensive to be practical (Cunningham *et al.*, "Phytoremediation of Contaminated Soils," *Trends Biotechnol.* 13: 393-397 (1995)).

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The ability of certain plants to grow in metal-containing or metal-contaminated soil, and to actively accumulate heavy metals in their tissues, has created an interest in using such plants to extract metals from soil. Growing plants, including crops, on contaminated soil to extract contaminants is referred to as phytoextraction. This method is particularly effective in arable contaminated soils because it causes little disruption or dispersal, while preserving soil fertility and landscapes.

Nickel is one of the most widely found, and technologically important metals. It is a natural constituent in all soils, being particularly high in concentration in certain types of soil and geological materials such as serpentine, lateritic serpentine, ultramafic and meteor-derived soils. Cobalt, another valuable metal, has chemical and geological characteristics very similar to nickel and is generally found in the same soils. Other metals that may be found in such soils include those of the platinum and palladium families such as palladium, rhodium, ruthenium, platinum, iridium, osmium and rhenium, and metals such as selenium, zinc and cadmium.

Sites containing serpentine, lateritic serpentine, ultramafic and meteor-derived soils and materials can be conventionally mined or cultivated with metal-accumulating plants. Using such plants to extract metals from mineralized (geogenic) soils is referred to as phytomining.

U.S. Patent No. 5,364,451 to Raskin *et al.*, is directed to a method of remediating polluted soils at a reduced cost. Raskin *et al.* remove metals from metal-rich soil by growing plants of the family Brassicaceae in the metal-rich soil. While Raskin *et al.* generally describe a variety of plants and a large number of metals that may be recovered, the examples mainly describe the recovery of chromium and lead from genetically altered plants. Thus, although promising, Raskin *et al.* offer little basis for an opportunity to proceed directly with soil phytomining or phytoextraction through plant growth or cultivation.

U.S. Patent No. 5,785,735 to Raskin *et al.*, is also directed to methods of remediating polluted soils. Raskin *et al.* remove metals from metal-rich soil by

growing crop and crop-related members of the plant family Brassicaceae in the metal-rich soil. The methods require the formation of a complex between the metal and a chelating agent added to the soil, the application of an electric field to the soil or a reduction in the pH of the soil. While Raskin *et al.* generally describe a variety of plants, the specification mainly describes the recovery of metals from genetically altered plants. Thus, again, Raskin *et al.* offer little basis for an opportunity to proceed directly with soil phytomining or phytoextraction through plant growth or cultivation.

Scientists recognize that increasing the pH of soil decreases the ability of farm crops to take-up heavy metals. U.S. Patent No. 5,711,784 to Chaney *et al.* reflects the belief in the art that reducing the pH of the soil "increases the phytoavailability of nickel and cobalt." As disclosed by Chaney *et al.*, a "reduced pH increases solubility, and optimizes the release of these metals for absorption by the roots and translocation to the above-ground tissues of the plant." However, reducing the pH of the soil also renders the metals more mobile and may allow for further contamination of the site. Therefore, cultivating plants which are hyperaccumulators of nickel, cobalt and other metals through phytoextraction or phytomining, is a desirable alternative as a means for recovering such metals.

Summary of the Invention

Accordingly, this invention relates to improved systems for recovering metals by phytomining or phytoextracting soils rich in metals.

The invention further relates to increasing nickel uptake by plants used in phytomining and phytoextraction by elevating the soil pH. Nickel is ultimately recovered from plant tissues at economically acceptable levels without further contaminating the nickel-containing site.

The invention further relates to lowering the pH in soils prior or subsequent to nickel recovery to collect, for example, cobalt or any other metal present in the metal-laden soil.

In a particular aspect of the invention, *Alyssum* plants are cultivated under favorable pH conditions to selectively accumulate certain metals relative to other metals.

The invention further relates to a method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:

(a) elevating or lowering the pH of the soil;
(b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one metal from the soil in above-ground tissue;

(c) elevating the pH of the soil if the pH was lowered in step (a) or lowering the pH of the soil if the pH was elevated in step (a); and

(d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.

The invention further relates to a method for recovering nickel from nickel-containing soil comprising:

(a) elevating the pH of the soil;
(b) cultivating at least one nickel-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel;

(c) harvesting said at least one plant; and
(d) recovering nickel from said harvested plant.

The invention further relates to a method for recovering cobalt from cobalt-containing soil comprising:

(a) lowering the pH of the soil;
(b) cultivating at least one cobalt-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is cobalt;

(c) harvesting said at least one plant; and
(d) recovering cobalt from said harvested plant.

The invention further relates to the identification of new hyperaccumulating species of *Alyssum* whereby collected plants are screened by comparing nickel-uptake by the plants to nickel-uptake by the bench-mark nickel-hyperaccumulator *A. murale* 103. These new metal-hyperaccumulating species, cultivated on nickel-containing soil, accumulate nickel in above-ground tissue at a concentration of 1.55% or greater by weight based on the gross dry weight of the tissue.

The invention further relates to seeds of the *Alyssum* plant species.

The invention further relates to pollen of the *Alyssum* plant species.

The invention further relates to plants that have all the physiological and morphological characteristics of the *Alyssum* plant species.

The invention further relates to propagation material of the *Alyssum* plant species.

The invention further relates to a method for decontaminating metal-containing soil, comprising cultivating at least one hyperaccumulator plant in metal-containing soil, whereby the concentration of metal in the above-ground plant tissue of the at least one hyperaccumulator plant exceeds the concentration of metal in the soil by a factor of at least 2.

Detailed Description of the Preferred Embodiments

In the present invention, it was discovered that certain metals can be selectively recovered from metal-rich soil using phytoextraction or phytomining techniques employing plants classified as hyperaccumulators of metals. By cultivating selected plants on metal-containing soil, the metals absorbed by the roots can be translocated to above-ground tissues, such as the stems, leaves, flowers and other leaf and stem tissues. This feature facilitates recovery of the metal extracted from the soil. Metal concentrations can be as high as about 5.0% in above-ground plant tissues, when leaves are included, which renders the metal recovery very economical. However, recovering metal in concentrations of less

than about 5.0%, such as about 4.0%, 3.0%, 2.5%, 1.0% or 0.1% remains useful. For example, a recovery of about 1.0% or more offers economic return for decontaminating polluted soil and for phytomining. A recovery of about 0.1% to about 1.0% of cobalt is sufficient to decontaminate polluted soil at a low cost, and a recovery of even less than about 0.1% of some metals can still effectively decontaminate polluted soils.

The invention further relates to a method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:

- (a) elevating or lowering the pH of the soil;
- (b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one metal from the soil in above-ground tissue;
- (c) elevating the pH of the soil if the pH was lowered in step (a) or lowering the pH of the soil if the pH was elevated in step (a); and
- (d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.

The invention further relates to a method for recovering nickel from nickel-containing soil comprising:

- (a) elevating the pH of the soil;
- (b) cultivating at least one nickel-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel;
- (c) harvesting said at least one plant; and
- (d) recovering nickel from said harvested plant.

The invention further relates to a method for recovering cobalt from cobalt-containing soil comprising:

- (a) lowering the pH of the soil;

(b) cultivating at least one cobalt-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is cobalt;

(c) harvesting said at least one plant; and

5 (d) recovering cobalt from said harvested plant.

The invention further relates to the identification of new hyperaccumulating species of *Alyssum* whereby collected plants are screened by comparing nickel-uptake by the plants to nickel-uptake by the bench-mark nickel-hyperaccumulator *A. murale* 103. These new metal-hyperaccumulating species,
10 cultivated on nickel-containing soil, accumulate nickel in above-ground tissue at a concentration of 1.55% or greater by weight based on the gross dry weight of the tissue.

The invention further relates to seeds of the *Alyssum* plant species.

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15 The invention further relates to plants that have all the physiological and morphological characteristics of the *Alyssum* plant species.

The invention further relates to propagation material of the *Alyssum* plant species.

20 The invention further relates to a method for decontaminating metal-containing soil, comprising cultivating at least one hyperaccumulator plant in metal-containing soil, whereby the concentration of metal in the above-ground plant tissue of the at least one hyperaccumulator plant exceeds the concentration of metal in the soil by a factor of at least 2, preferably by a factor of 2, 3 or 4.

25 In a preferred aspect of the invention, nickel is selectively accumulated by growing one or more nickel-hyperaccumulating plants in metal-rich, e.g., nickel-rich, soil and elevating the pH of the soil. The pH of the soil may be elevated before, during or after the plants are cultivated. Preferably, the pH is elevated prior to plant cultivation. Thus, the invention relates to the surprising discovery that raising the pH of the metal-rich soil favors nickel accumulation in plant
30 tissue over other metals. The soil pH can then be lowered to selectively

accumulate, in the plant tissue, other metals such as cobalt. The preferred pH will depend, *inter alia*, upon the particular metal and the soil. For example, the preferred pH for nickel extraction ranges between about 6.3 and about 7.0 when the soil is a serpentine soil or when the soil contains high iron oxide levels. The most preferred pH ranges from about 6.3 to about 6.7. However, when the iron oxide level is low, a more alkaline pH may be used.

Cobalt extraction is also affected by the soil chemistry. For example, the most preferred pH for cobalt extraction is about 5.5 when aluminum and/or manganese are present in the soil. For metal extraction in general, the preferred pH ranges between about 5.5 and about 7.0.

Soil pH can be raised and lowered with bases and acids. Such bases and acids may be either naturally occurring or synthetic. To raise the pH, bases such as limestone (calcitic (CaCO_3) or dolomitic (CaMgCO_3)), lime (CaO), hydrated lime (Ca(OH)_2), industrial, municipal or agricultural alkaline by-products that contain any of the above bases or a limestone equivalent, or the like can be used. The phrase "limestone equivalent" is intended to encompass bases that have the same alkalinity as limestone. To lower the pH, acids such as organic and inorganic acids can be used. Examples of such organic and inorganic acids include acetic acid, aqueous hydrogen chloride, aqueous sulfuric acid, sulfur, ammonium, urea-containing fertilizers, nitric acid, sulfide minerals, including, but not limited to, pyrite, and the like.

The amount of base or acid to add depends upon the existing pH of the soil and the soil chemistry. Methods used to determine the amount include, but are not limited to, adding acid or a base, such as CaCO_3 , to the soil sample and measuring the resulting pH, then drawing a pH response curve to extrapolate the amount needed to obtain the desired pH.

After cultivation, the hyperaccumulator plant is harvested in a conventional fashion, i.e., by cutting the plant at soil level. The harvested materials are then left to dry in the field in the manner in which hay is dried. Alternatively, the harvested materials are dried in much the same fashion that

alfalfa is dried, so as to remove most of the water present in the plant tissue by forced heated air drying. After drying, the plant tissue is collected by normal agricultural practices of hay-making, incinerated and reduced to an ash with or without energy recovery. Alternatively, the dried plant material may be hydrolyzed with concentrated acid to produce sugars and the metals recovered according to U.S. Patent Nos. 5,407,817, 5,571,703 and 5,779,164. The sugars may then be fermented to produce ethanol.

The resulting dried plant material may alternatively be further treated by known roasting, sintering or smelting methods which allow the metals in the ash or ore to be recovered according to conventional metal refining methods such as acid dissolution and electrowinning.

Conventional smelting, roasting and sintering temperatures from about 260°C to about 1000°C are sufficient to combust the dried plant material to oxidize and vaporize the organic material present and to prevent dioxin accumulation during incineration. The preferred temperature is sufficient to remove the organic carbon to free the ash. The most preferred temperature is about 1000°C. The process leaves a residue of the accumulated metal with few contaminants known to interfere with metal refining. Further, it is expected that the concentration of other components in the ash will be much lower than with conventional mined ore concentrates. For example, serpentine laterite ores generally contain over 10,000 ppm (1%) Fe whereas a biomass obtained using phytomining techniques only contains about 100-500 ppm (0.01 - 0.05%) Fe.

By definition, nickel-hyperaccumulating plants accumulate at least about 1000 mg of nickel per 1 kg dry weight of plant tissue (obtained from a plant grown in soil where the plant naturally occurs). Similarly, cobalt-hyperaccumulating plants are defined as plants that accumulate at least about 1000 mg of cobalt per 1 kg dry weight of plant tissue (obtained from a plant grown in soil where the plant naturally occurs). However, zinc- and manganese-hyperaccumulators are defined as plants that accumulate at least about 10,000 mg of zinc and manganese, respectively, per 1 kg dry weight of plant tissue (obtained

from a plant grown in soil where the plant naturally occurs). Finally, cadmium-hyperaccumulators are defined as plants that accumulate at least about 100 mg cadmium per 1 kg dry weight of plant tissue (obtained from a plant grown in soil where the plant naturally occurs).

5 By screening a wide variety of plants, those of the *Alyssum* genus (Brassicaceae family) have been identified as hyperaccumulators of nickel. These plants also naturally accumulate cobalt and may accumulate metals such as Zn, Mn and Cd, and metals from the platinum and palladium families including Pd, Rh, Ru, Pt, Ir, Os and Re.

10 More specifically, plants which naturally concentrate nickel in above-ground tissues and generally exhibit an enhanced uptake of cobalt and other metals include members of the section *Odontarrhena* of the genus *Alyssum*.

The metals accumulate in nickel-hyperaccumulating *Alyssum* plant species when the plants are grown in contaminated soils. Some 48 taxa within the section

15 *Odontarrhena* of the genus *Alyssum* are known to be hyperaccumulators of nickel.

These include the following species: *A. akamasicum*, *A. alpestre*, *A. anatolicum*, *A. callichroum*, *A. cassium*, *A. chondrogynum*, *A. cilicicum*, *A. condensatum*, *A. constellatum*, *A. crenulatum*, *A. cypricum*, *A. davisianum*, *A. discolor*, *A. dubertretii*, *A. eriophyllum*, *A. euboeum*, *A. floribundum*, *A. giosnanum*, *A. huber-morathii*, *A. janchenii*, *A. markgrafii*, *A. masmenaeum*, *A. obovatum*, *A. oxycarpum*, *A. penjwinensis*, *A. pinifolium*, *A. pterocarpum*, *A. robertianum*, *A. samariferum*, *A. singarense*, *A. smolikanum*, *A. syriacum*, *A. trapeziforme*, *A. troodii*, *A. virgatum*, *A. murale*, *A. pintodasilvae* (also known as *A. serpyllifolium* var. *lusitanicum*), *A. serpyllifolium*, *A. malacitanum* (also known as *A. serpyllifolium* var. *malacitanum*), *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A. bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum* and *A. caricum* as well as newly discovered species such as *A. corsicum* G16, *A. murale* G69 and *A. murale* G82. These species were deposited on November 6, 1998, under the provisions of the Budapest Treaty at the American Type Culture Collection,

10801 University Blvd., Manassas, VA 20110-2209, and assigned ATCC nos. 203436, 203437 and 203438, respectively.

Species of *Alyssum* that naturally accumulate nickel in amounts of up to 20% greater than any known *Alyssum* hyperaccumulator have been isolated. Species *A. murale* G49, *A. murale* G54, *A. murale* G69 and *A. murale* G82 isolated in Greece and species *A. corsicum* G16 isolated in Turkey all accumulate nickel in amounts greater than the known species *A. murale* 103 which accumulates nickel such that nickel makes up 1.14% by dry weight of a plant shoot from a test field of serpentine soil. The new hyperaccumulators accumulate nickel in amounts such that 1.55-1.60% by dry weight of the shoot is nickel. The results of nickel accumulation of these five new accumulators relative to the benchmark accumulator *A. murale* 103 is shown in Example 4.

About 250 other plant taxa, including those of tropical origin, have been shown to accumulate quantities of nickel and other metals. However, many of these plants do not exceed about 10,000 mg of metal per kg of plant tissue dry weight. Other metal-accumulating plants includes species of the genus *Cyanotis* such as *Cyanotis longifolia*; species of the genus *Bulbostylis* such as *Bulbostylis mucronata*; species of the genus *Combretum* such as *Combretum decandrum*; species of the genus *Crassula* such as *C. alba*, *C. vaginata* and *C. argyrophylla*; species of the genus *Clethra* such as *Clethra barbinervis*; plants from the Cunoniaceae family such as species of the genus *Geissois* including *G. intermedia*, *G. magnifica*, *G. montana*, *G. pruinosa*, *G. trifoliata* and *G. racemosa*; species of the genus *Argophyllum*; members of Brassicaceae family such as species of the genus *Thlaspi* such as *Thlaspi caerulescens*, *Thlaspi montanum* var. *montanum* and *Thlaspi montanum* var. *siskiyounense*; species of the genus *Serpentine* such as *Serpentine polygaloides*; species of the genus *Sebertia* such as *Sebertia acuminata*; species of the genus *Hybanthus* such as *Hybanthus floribundas*; species of the genus *Psychotria* such as *Psychotria douarrei*; species of the genus *Rinorea* such as *Rinorea bengalensis*; species of the genus *Pearsonia* such as *Pearsonia metallifera*; species of the genus *Sebertia*

such as *Sebertia acuminata*; and species of the following genera: *Homalium*, *Myristica*, *Trichospermum*, *Planchonella* and *Peltaria*. Additional plants include, but are not limited to, *Streptanthus polygaloides*, *Berkheya coddii*, *Phyllanthus palawanensis*, *Dichapetalum gelonioides* ssp. *tuberculatum* and *Stackhousia tryonii*.

Additional metal hyperaccumulators are listed below:

ACANTHACEAE

Blepharis acuminata, *Justicia lanstykii*, *Lophostachys villosa*, *Phidiasia lindavii*, *Ruellia geminiflora*

ADIANTACEAE

Adiantum sp.

ANACARDIACEAE

Rhus wildii

ASTERACEAE

Berkheya coddii, *Chromolaena* sp. cf. *meyeri*, *Dicoma niccolifera*, *Gochnatia crassifolia*, *G. recurva*, *Koanophyllon grandiceps*, *K. prinodes*, *Leucanthemopsis alpina*, *Pentacalia*, *Senecio* *Senecio pauperculus*, *Shafera platyphylla*, *Solidago hispida*

BORAGINACEAE

Heliotropium sp.

BRASSICACEAE

Bornmuellera, *Cardamine resedifolia*, *Cochlearia aucheri*, *C. sempervivum*, *Peltaria emarginata*, *Streptanthus polygaloides*

BUXACEAE

Buxus

CAMPANULACEAE

Campanula scheuchzeri, *Arenaria*, *Minuartia laricifolia*, *M. verna*

CLUSIACEAE

Garcinia bakeriana, *G. polyneura*, *G. revoluta*, *G. ruscifolia*

CONVOLVULACEAE

Merremia xanthophylla

CUNONIACEAE

Pancheria engleriana

DICHAPETALACEAE

Dichapetalum gelonioides and ssp. *tuberculatum* and ssp. *andamanicum*

DIPTEROCARPACEAE

5 *Shorea tenuiramulosa*

ESCALLONIACEAE

Argophyllum grunowii, *A. laxum*

EUPHORBIACEAE

10 *Baloghia* sp., *Bonania*, *Cleidion viellardii*, *Cnidoscolus* sp. cf. *bahianus*,
Euphorbia, *Gymnanthes recurva*, *Leucocroton*, *Phyllanthus*, *Sapium*
erythrospermum, *Savia*

FABACEAE

Anthyllis sp., *Pearsonia metallifera*, *Trifolium pallescens*

FLACOURTIACEAE

15 *Casearia silvana*, *Homalium*, *Xylosma*

JUNCACEAE

Luzula lutea

MELIACEAE

Walsura monophylla

20 MYRISTICACEAE

Myristica laurifolia

MYRTACEAE

Mosiera araneosa, *M. ekmanii*, *M. x miraflorensis*, *M. ophiticola*, *Psidium*
araneosum, *P. havanense*

25 OCHNACEAE

Brackenridgea palustris and ssp. *foxworthyi* and ssp. *kjellbergii*, *Ouratea nitida*,
O. striata

OLEACEAE

Chionanthus domingensis

30 ONCOTHECACEAE

Oncotheca balansae

POACEAE

Trisetum distichophyllum

RANUNCULACEAE

Ranunculus glacialis

5 RUBIACEAE

Ariadne shaferi ssp. *shaferi* and ssp. *moaensis*, *Mitracarpus* sp., *Phyllomelia coronata*, *Psychotria clementis*, *P. costivenia*, *P. douarrei*, *P. glomerata*, *P. osseana*, *P. vanhermanii*, *Rondeletia*

SAPOTACEAE

10 *Planchonella oxyedra*, *Sebertia acuminata*

SAXIFRAGACEAE

Saxifraga

SCROPHULARIACEAE

Esterhazyia sp. and *Linaria alpina*

15 STACKHOUSIACEAE

Stackhousia tryonii

TILIACEAE

Tetralix brachypetalus, *T. cristalensis*, *T. jaucoensis*, *T. moaensis*, *T. nipensis*, *Trichospermum kjellbergii*

20 TURNERACEAE

Turnera subnuda

VELLOZIACEAE

Vellozia sp.

VIOLACEAE

25 *Agatea deplanchei*, *Hybanthus*, *Rinorea bengalensis*, *R. javanica*, *Rinorea* sp.

ACERACEAE

Acer pseudoplatanus

BRASSICACEAE

30 *Cardaminopsis halleri*, *Thlaspi avalanum*, *T. brachypetalum*, *T. caerulescens*, *T. ochroleucum*, *T. rotundifolium* subsp. *cepaefolium*, *T. praecox*, *T. stenopterum*, *T. tatrense*

CARYOPHYLLACEAE

Minuartia verna, *Polycarpaea synandra*

CISTACEAE

Cistus incanus ssp. *creticus*

DICHAPETALACEAE

5 *Dichapetalum gelonioides*

PLUMBAGINACEAE

Armeria maritima var. *halleri*

POACEAE

Agrostis stolonifera, *A. tenuis*, *Arrhenatherum elatius*, *Festuca ovina*

10 POLYGONACEAE

Rumex acetosa

VIOLACEAE

Viola calaminaria

AMARANTHACEAE

15 *Pandiana metallorum*, *Celosia trigyna*

ASTERACEAE

Anisopappus chinensis, *A. davyi*, *Gutenbergia pubescens*, *Millotia myosotidifolia*, *Vernonia petersii*

20 CARYOPHYLLACEAE

Minuartia verna ssp. *hercynica* and *Silene cobalticola*

COMMELINACEAE

Commelina zigzag and *Cyanotis longifolia*

CONVOLVULACEAE

25 *Ipomoea alpina*

CRASSULACEAE

Crassula alba and *C. vaginata*

CYPERACEAE

Ascolepis metallorum, *Bulbostylis cupricola*, *B. pseudoperennis*

30 EUPHORBIACEAE

Monadenium cupricola and *Phyllanthus williamoides*

FABACEAE

Crotalaria cobalticola and *Vigna dolomitica*

IRIDACEAE

Gladiolus gregarius

5

LAMIACEAE

Aeollanthus subacaulis var. *linearis*, *A. homblei*, *A. saxatilis*, *A. subacaulis* var. *ericoides* and var. *linearis*, *Becium grandiflorum* var. *vanderystii*, *Haumaniastrum homblei*, *H. katangense*, *H. robertii*, *H. rosulatum*

10

MALVACEAE

Hibiscus rhodanthus

PINACEAE

Abies balsamea

POACEAE

15

Eragrostis racemosa, *Rendlia altera*, *Sporobolus congoensis*

PTERIDACEAE

Actinopteris sp.

SCROPHULARIACEAE

20

Alectra sessiliflora var. *senegalensis*, *Buchnera henriquesii*, *Crepidorhopalon tenuis*, *C. perennisa*, *Sopubia mannii*, *S. metallorum*, *S. neptunii*, *Striga hermontheca*

TILIACEAE

Triumfetta dekindtiana, *T. digitata*, *T. welwitschii* var. *descampii*

VELLOZIACEAE

25

Xerophyta retinervis var. *equisetoides*

APOCYNACEAE

Alyxia rubricaulis

CELASTRACEAE

Maytenus bureaviana, *M. pancheriana*, *M. sebertiana*

30

CLUSIACEAE

Garcinia amplexicaulis

MYRTACEAE

Eugenia clusioides

PROTEACEAE

Beaupreopsis paniculata, *Macadamia angustifolia*, *M. neurophylla*

ASTERACEAE

Haplopappus fremontii, *Machaeranthera glabriuscula*, *M. ramosa*, *M. venusta*

5

BRASSICACEAE

Stanleya pinnata, *S. bipinnata*

CHENOPODIACEAE

Atriplex confertifolia

10

LECYTHIDACEAE

Lecythis ollaria

LEGUMINOSAE

Acacia cana, *Astragalus bisulcatus*, *A. osterhoutii*, *A. pattersonii*, *A. pectinatus*,
A. racemosus, *Neptunia amplexicaulis*

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RUBIACEAE

Morinda reticulata

SCROPHULARIACEAE

Castilleja chromosa

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The metals accumulated include nickel, cobalt, barium, gold, beryllium, mercury, molybdenum, copper, arsenic, selenium, antimony, manganese, silver, thallium, tin, lead, rubidium, chromium, cerium, vanadium, cesium, uranium, plutonium, strontium, yttrium, technetium, iridium, ruthenium, palladium, rhodium, platinum, osmium, rhenium, zinc and cadmium.

25

Metal sequestration can be improved by optimizing soil calcium concentration, using ammonium-containing or ammonium-generating fertilizers rather than other nitrate-containing fertilizers, and by applying chelating agents to the soil in which the hyperaccumulator plants are grown.

30

Alyssum species which hyperaccumulate metals such as nickel and cobalt evolved in nickel-rich ultramafic and serpentine soils which have low soil calcium and a low Ca:Mg ratio. It is now known that the presence of extremely low and extremely high calcium concentrations in soil inhibits nickel

hyperaccumulation by *Alyssum*. See PCT/US97/15109. Acceptable calcium concentrations in soil range from about 0.128 mM to about 5.0 mM. In terms of percentages, an acceptable calcium concentration in soil ranges from about 2% to about 80% of the exchangeable cations. A preferable range is from about 10% to about 80% of the exchangeable cations. The most preferred range is from about 30% to about 70% of the exchangeable cations. Such ranges can be achieved, if necessary, by adding calcium-containing agents to the soil such as limestone. In addition, gypsum could be added to the soil to raise the exchangeable calcium of the soil to benefit nickel accumulation.

The presence of intermediate concentrations of calcium, i.e., between about 0.128 mM and about 5.0 mM, increases nickel uptake whereas calcium values of about 0.128 mM and below, or about 5 mM and above, decrease nickel uptake. Combined with an exchangeable Ca:Mg ratio of between about 0.16 and about 0.40, much lower than recommended, an additional increase in nickel concentration in plant tissues is observed. By "exchangeable Ca:Mg ratio" is intended the ratio of extractable calcium and magnesium in the soil.

Although hyperaccumulators such as *Alyssum* have developed the ability to hyperaccumulate metals in above-ground tissues, fertilizer supportive of growth, particularly in polluted soil, can be used as an additive to increase hyperaccumulation. Ammonium fertilizers localize acidification adjacent to the root which aids hyperaccumulation of various metals such as Ni, Zn, Cd, Co, etc. The use of ammonium fertilizers *per se* is well-known, and acceptable fertilizers and protocols can be readily determined with no more than routine experimentation, by those of ordinary skill in the art. Other additives include, but are not limited to, nutrients such as phosphate which helps to maximize the yield of nickel, for example.

Another possible additive to the contaminated soil is a metal chelating agent. Metal chelates are commonly used in agriculture and occur naturally in living cells. The addition of chelating agents, such as nitrolotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA),

ethyleneglycol-bis-(p-aminoethylether-N, N-tetraacetic acid) or any of a variety of amino-acetic acids known to those of ordinary skill in the art as chelating agents, to the soil to be phytomined or phytoextracted improves the movement of soil metals to root surfaces for uptake and translocation into above-ground tissues. Preferred chelating agents are NTA or EDTA. Typically, chelating agents will be added at a concentration ranging from about 0.5 to about 10 millimoles per kg soil. As with the use of fertilizers, the optimum concentration of chelating agents can be readily determined with no more than routine experimentation. Chelating compounds which chelate nickel in the presence of high soil levels of Fe, Mg and Ca selectively increase nickel uptake by hyperaccumulator plants.

The following examples are illustrative, but not limiting, of the methods of the present invention. Other suitable modifications and adaptations of the variety of conditions normally encountered which are obvious to those skilled in the art are within the spirit and scope of the present invention.

Examples

Example 1

A. murale 103 plants were grown in sets of two for 120 days in 19 pot-sets (4 L) of contaminated or serpentine soils (Mg-nitrate was leached out) without acidification, the first pot in a set, and with acidification, the second pot in a set. Water was maintained near field capacity by daily watering with deionized water. The plants were cultivated at a temperature of about 28°C during the day and about 20°C at night. The soils were acidified using nitric acid and the pH was raised using powdered reagent-grade CaCO₃. The soils included serpentine soils rich in nickel (containing from about 100 to about 5000 ppm nickel) obtained from southwest Oregon (soils 3-19), nickel-refinery contaminated Welland loam from Port Colborne, Ontario (soil 1) and nickel-refinery contaminated Quarry muck from Port Colborne, Ontario (soil 2). Fertilizers containing, *inter alia*, nickel, potassium, sulfur and phosphorous, were added to optimize plant growth.

Table 1 shows the results of the experiment in contaminated soil.

Table 1

Soil	TRT	Final pH	Yield	Ni	Co	Mn	Zn	Cu	Fe
			g shoot dry matter/ pot	mg/kg					
1	2	5.16	27.4	9150	119	82.4	117	15.0	58
1	6	4.96	22.7	4220	84.7	145.6	180	19.5	64
2	2	6.04	40.9	4570	5.9	20.9	99.0	4.0	68
2	6	5.40	28.8	2150	7.1	63.0	142	6.5	82
3	2	6.26	21.5	6370	19.9	68.8	61.5	3.5	160
3	6	5.38	19.7	6480	308	680	65.9	5.5	260
4	2	5.61	19.6	12400	56.5	181	88.0	4.0	332
4	6	5.21	15.6	8560	377	140	135	5.0	345
5	2	5.88	24.0	1860	6.0	53.0	252	3.2	137
5	6	5.32	21.1	1220	9.8	153	379	3.5	121
6	2	6.03	24.5	4580	14.6	84.2	61.2	5.2	183
6	6	5.42	27.2	5040	58.5	227	70.3	5.5	195
7	2	5.54	23.3	5750	36.3	134	83.7	5.0	250
7	6	5.28	23.2	4870	86.8	272	77.9	5.5	274
8	2	5.77	21.1	9630	28.8	130	52.6	4.0	223
8	6	5.21	17.5	7180	94.0	291	74.9	4.8	221
9	2	6.12	22.1	9770	38.7	122	69.6	4.8	240
9	6	5.62	22.5	9100	196	532	69.7	5.2	273
10	2	6.25	20.0	12900	31.2	109	79.3	2.5	318
10	6	5.76	19.3	11500	182	774	93.5	3.2	412
11	2	5.72	32.8	8460	37.3	148	75.5	5.0	266
11	6	5.35	24.3	6010	136	339	93.6	4.8	230
12	2	6.54	20.3	8070	29.0	84.4	74.0	3.5	222
12	6	5.78	18.4	8240	86.0	186	66.5	3.2	178
13	2	6.34	18.8	11000	16.2	39.1	51.8	2.2	186

-21-

Soil	TRT	Final pH	Yield	Ni	Co	Mn	Zn	Cu	Fe
			g shoot dry matter/ pot	mg/kg					
13	6	5.87	19.6	9970	36.0	103	56.6	2.8	181
14	2	5.68	21.3	9150	67.0	331	65.8	4.8	278
14	6	4.84	13.3	5820	313	957	86.0	4.8	567
15	2	6.04	19.4	7620	30.5	142	69.8	4.8	365
15	6	5.94	23.7	6110	463	820	88.6	4.8	220
16	2	6.07	21.0	3090	47.4	128	89.1	6.8	172
16	6	5.41	18.2	3560	225	563	105	8.0	267
17	2	6.02	20.6	9080	37.5	124	114	3.8	256
17	6	5.63	23.9	7940	262	973	127	4.2	252
18	2	5.99	19.4	11600	35.3	127	68.5	3.0	440
18	6	5.53	15.4	9500	204	908	116	4.2	548
19	2	5.59	21.8	436	19.1	259	92.4	7.8	190
19	6	5.11	19.5	584	72.4	929	112	8.8	156

"TRT" = treatment. In treatment 2, the soil pH was not adjusted. In treatment 6, the soil pH was acidified.

As illustrated in Table 1, the plants grown on soils of less acidic pH generally accumulated far greater amounts of nickel than the plants grown on more acidic soils. In addition, plants taking up larger amounts of nickel on less acidic soils accumulated smaller amounts of other metals such as cobalt, manganese and zinc which are commonly found in lower concentrations in shoots after soil pH is raised.

Example 2

To validate the above example and to obtain optimization, *Alyssum* plants were grown on nickel-refinery contaminated Welland loam (soil 1), wherein the pH was elevated by applying limestone (Table 2). The plants were also grown on

nickel-refinery contaminated Quarry muck (soil 2) and serpentine soils (soils 3-11) (Table 3). The same cultivation conditions recited in Example 1 were used in Example 2.

Table 2

5 Effect of phosphate, pH and Ca:Mg variation on geometric mean shoot yield and micronutrient composition of two *Alyssum* species grown on nickel-refinery contaminated Welland loam (soil 1) for 120 days.

Soil	TRT	Yield	Ni	Co	Mn	Zn
		g/pot	g/kg	mg/kg	mg/kg	mg/kg
1	1	6.68 b*	7.61 a	127 a	23.7 e	157 fg
Phosphate Series:						
1	3	7.82 ab	5.94 bc	118 ab	72.8 c	209 ab
1	2	9.78 ab	5.49 cd	109 bcd	59.3 d	170 def
1	4	8.71 ab	6.40 b	114 a-d	66.7 cd	178 c-f
1	5	8.03 ab	5.97 bc	98.8 d	60.8 cd	169 def
pH Series:						
1	6	8.14 ab	3.93 e	132 a	177 a	217 a
1	7	7.46 ab	4.93 d	119 ab	99.8 b	183 b-e
1	2	9.78 ab	5.49 cd	109 bcd	59.3 d	170 deg
1	8	10.4 a	8.47 a	101 cd	19.1 f	142 g
Ca:Mg Series:						
1	9	9.22 ab	6.10 bc	119 ab	67.3 cd	168 ef
1	2	9.78 ab	5.49 cd	109 bcd	59.3 d	170 def
1	10	7.80 ab	5.55 cd	117 abc	64.7 cd	198 abc
1	11	8.72 ab	5.85 bc	120 ab	69.8 cd	195 a-d

25 *a-g indicate means followed by the same letter are not significantly different at the $P < 0.05$ level according to the Duncan-Walker K-ratio t-test.

"TRT" = treatment

Table 3

Effect of soil treatments on soil pH and micronutrient composition of *Alyssum murale* and *Alyssum corsicum* grown on nickel-refinery contaminated Welland loam (soil 1), nickel-refinery contaminated Quarry muck (soil 2) and serpentine soils (soils 3-11) for 120 days.

Soil	TRT	Final pH	Cu	Zn	Co	Ni	Mn	Fe
			mg/kg	mg/kg	mg/kg	g/kg	mg/kg	mg/kg
1		5.47	11.0	156	136	8.13	39.2	67.6
<u>Phosphate Series (phosphate added to the soil in kg/ha by the addition of phosphate-containing fertilizer):</u>								
3	0 P	5.23	15.0	179	99.1	7.58	56.2	49.6
2	100 P	5.18	16.0	131	102	7.34	59.7	50.1
4	250 P	5.24	14.5	133	82.2	7.37	56.8	56.4
5	500 P	5.13	14.5	129	73.8	6.50	53.1	50.8
<u>pH Series (soil was acidified using nitric acid for "Lo pH" and "MLo pH"):</u>								
6	Lo pH	4.99	19.2	192	91.0	4.16	129	53.1
7	MLo pH	5.18	16.8	160	104	5.77	81.2	64.0
2	As is pH	5.18	16.0	131	102	7.34	59.7	50.1
8	Limed	5.57	10.1	102	71.1	9.28	19.9	57.6
<u>Ca:Mg Ratio Series:</u>								
9	1.0 Ca	5.25	17.0	134	108	7.32	65.0	55.0
2	0 Ca/Mg	5.18	16.0	131	102	7.34	59.7	50.1
10	2.5 Mg	5.13	17.4	152	90.4	6.75	48.9	53.0
11	5.0 Mg	5.04	16.2	149	87.6	5.71	54.8	67.1

"TRT" = treatment

"MLo pH" = medium-low pH

The soil designations correspond to the soil designations in Example 1.

The "pH series" experiments demonstrate that the application of limestone increases the uptake of nickel in *Alyssum* so that plant tissues accumulate an increased concentration of nickel.

Example 3

5 The results show an increase in the geometric mean of nickel uptake in
plant tissue by liming *Alyssum* plants cultivated on nickel-refinery contaminated
Quarry muck (soil 2) (Table 4) and on nickel-refinery contaminated Welloam
loam (soil 1), nickel-refinery contaminated Quarry muck (soil 2) and selected
serpentine soils (soils 3-11) (Table 5) from Example 1. The cultivation
10 conditions were the same as those for Examples 1 and 2.

Table 4

Effects of soil treatments on the mean concentrations of elements in whole shoots and shoot yield of *Alyssum murale* and *Alyssum corsicum* grown on nickel-refinery contaminated Quarry muck (soil 2) for 60 days.

Soil	TRT	Treatment	Shoot Yld	Shoot Ni	Shoot Co	Shoot MN
			g/pot	g/kg	mg/kg	mg/kg
2	1	None	8.46 d*	3.33 abc	8.62 ab	27.9 bc
<u>Phosphate Series:</u>						
2	3	0 P	10.78 a-d	3.24 bc	5.50 b	15.0 bc
2	2	100 P	12.09 a	3.23 bc	5.75 ab	14.5 bc
2	4	250 P	11.53 abc	3.76 a	5.50 b	18.6 bc
2	2	500 P	11.86 ab	3.30 abc	6.38 ab	27.7 bc
<u>pH Series:</u>						
2	6	Lo pH	12.01 ab	1.48 e	10.25 a	59.8 a
2	7	Med pH	9.44 bcd	2.12 d	6.12 ab	29.0 b
2	2	As is pH	12.09 a	3.23 bc	5.75 ab	14.5 bc
2	8	Limed	11.14 abc	3.72 ab	5.88 ab	13.3 c
<u>Ca:Mg Series:</u>						
2	9	Ca	9.08 cd	3.42 abc	6.38 ab	16.3 bc
2	2	As is Ca	12.09 a	3.23 bc	5.75 ab	14.5 bc
2	10	Med Mg	11.66 ab	3.03 c	4.62 b	24.9 bc
2	11	Hi Mg	9.98 a-d	2.94 c	5.25 b	23.3 bc

*a-e indicate means followed by the same letter are not significantly different at the $P < 0.05$ level according to the Duncan-Walker K-ratio t-test.

"TRT" = treatment

Table 5

Effect of altering nickel-refinery contaminated Welland loam (soil 1), nickel-refinery contaminated Quarry muck (soil 2) and serpentine soils (soils 3-11) by adding phosphate, adjusting the pH or adjusting the Ca:Mg ratio on soil pH, mean yield and micronutrient composition of shoots of *Alyssum* species grown for 120 days (GM designates geometric mean).

Soil	TRT	Final pH	GM-Yield	GM-Ni	GM-Co	GM-Mn	GM-Zn	GM-Fe	GM-Cu
			g/pot	mg/kg					
1	None	6.34	20.2	5460	7.6	11.9	151	61	4.8
Phosphate Treatments (phosphate added to the soil in kg/ha by the addition of phosphate-containing fertilizer):									
3	0 P	6.09	41.6	4400	5.8	16.5	152	56	4.2
2	100 P	6.05	42.7	4120	5.7	18.6	126	57	4.5
4	250 P	6.07	49.9	4120	5.1	21.4	143	57	4.8
5	500 P	5.98	46.4	3800	5.1	22.9	139	54	4.2
pH Treatments (soil was acidified using nitric acid for "Lo pH" and "Med-pH"):									
6	Lo pH	5.44	32.2	2010	6.8	50.5	153	68	6.4
7	Med-pH	5.76	36.1	2700	4.5	21.0	143	60	4.8
2	As is pH	6.05	42.7	4120	5.7	18.6	126	57	4.5
8	Limed	6.20	40.5	4520	6.3	15.8	137	55	4.1
Ca:Mg Treatments:									
9	0.0 Ca	6.13	38.6	4510	6.3	16.2	135	57	4.8
2	1.0 Ca	6.05	42.7	4120	5.7	18.6	126	56	4.5
10	2.5 Mg	5.98	39.0	4410	5.9	16.2	146	63	4.6
11	5.0 Mg	5.91	44.0	4260	5.8	18.3	158	58	4.6

"TRT" = treatment

The soil designations correspond to the soil designations in Example 1.

Example 4 - Novel Hyperaccumulators

The concentration of elements in the shoots of *Alyssum* species grown on a field of serpentine colluvial soil in Josephine County, Oregon, are shown in Table 6 below.

Table 6

Row	Species	Genotype	Block	Zn	P	Cu	Co	Ni	Mn	Fe	Mg	Ca	K
139	<i>A. corsicum</i>	16	1	137	5.01	9	14	13400	53	53.8	5.52	20.9	43.3
483	<i>A. corsicum</i>	16	2	141	4.08	8	16	17500	32	755	5.99	24.2	44.3
129	<i>A. murale</i>	49	1	99	4.80	7	12	14100	41	397	3.98	32.1	41.4
325	<i>A. murale</i>	49	2	106	4.63	8	16	17100	46	455	5.32	31.7	41.7
135	<i>A. murale</i>	54	1	119	4.18	5	13	15600	53	927	4.02	25.8	44.5
143	<i>A. murale</i>	69	1	165	5.78	5	16	16700	53	380	4.52	17.3	38.8
553	<i>A. murale</i>	69	2	191	4.97	6	15	13400	45	616	5.66	25.4	6.16

The elements are present in mg/kg amounts.

Whole shoots or side branch samples containing stems and leaves were collected from pots or the field for each genotype, dried in forced air drying ovens and ground with a non-contaminating mill to less than about 0.1 mm. The ground samples were then placed in a borosilicate beaker and ashed at 480°C overnight. Nitric acid was added to dissolve the resultant ash which was then heated until dry on a hot plate. Hydrochloric acid (3.0 M) was added and the beaker was refluxed for two hours to determine recovered nickel concentration. Concentrations of nickel were measured by an inductively coupled argon plasma emission spectrometer. Low concentrations were measured by atomic absorption spectrometry.

This invention has been described in specific detail with regard to specific plants and methods for increasing metal, such as nickel, uptake via phytomining or phytoextraction. Except where necessary for operability, no limitation to these

5 specific materials is intended nor should such a limitation be imposed on the claims appended hereto. From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions without undue experimentation. All patents, patent applications and publications cited herein are incorporated by reference in their entirety.

What Is Claimed Is:

1. A method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:
 - (a) elevating the pH of the soil; and
 - (b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate said at least one metal from the soil in above-ground tissue.
2. The method of claim 1, wherein said at least one metal is nickel.
3. The method of claim 1, wherein the pH of the soil is elevated by adding to the soil at least one agent that results in an increase in the soil pH.
4. The method of claim 3, wherein the at least one agent that results in an increase in the soil pH is selected from the group consisting of limestone, dolomitic limestone, lime, hydrated lime, limestone equivalents and mixtures thereof.
5. The method of claim 1, further comprising after said cultivating:
 - (c) lowering the pH of the soil; and
 - (d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.
6. The method of claim 5, wherein the at least one second metal is selected from the group consisting of: cobalt, palladium, rhodium, ruthenium, platinum, iridium, osmium, rhenium and mixtures thereof.

7. The method of claim 5, wherein the pH of the soil is lowered by adding to the soil at least one agent that results in a decrease of the soil pH.
8. The method of claim 1, wherein said at least one plant is an *Alyssum* plant.
- 5 9. The method of claim 8, wherein said *Alyssum* plant is selected from the group consisting of: *A. murale*, *A. pintodasilvae*, *A. serpyllifolium*, *A. malacitanum*, *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A. bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum*, *A. caricum* and mixtures thereof.
- 10 10. A method for recovering nickel from nickel-containing soil comprising:
 - (a) elevating the pH of the soil;
 - (b) cultivating at least one nickel-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel;
 - 15 (c) harvesting said at least one plant; and
 - (d) recovering nickel from said harvested plant.
11. The method of claim 10, wherein the soil has a calcium concentration of between about 20-80%.
- 20 12. The method of claim 10, wherein the nickel is recovered by drying and combusting the harvested plant to oxidize and vaporize organic material present.
13. The method of claim 10, wherein said at least one plant is an *Alyssum* plant.

5 14. The method of claim 13, wherein said *Alyssum* plant is selected from the group consisting of: *A. murale*, *A. pintodasilvae*, *A. serpyllifolium*, *A. malacitanum*, *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A. bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum*, *A. caricum* and mixtures thereof.

15. The method of claim 14, wherein said *Alyssum* plant is selected from the group consisting of: *A. corsicum* G16, *A. murale* G69, *A. murale* G82 and mixtures thereof.

10 16. The method of claim 10, wherein at least about 2.5% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel.

17. The method of claim 16, wherein at least about 3.0% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel.

18. The method of claim 17, wherein about 4.0% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel.

15 19. A method for recovering cobalt from cobalt-containing soil comprising:
(a) lowering the pH of the soil;
(b) cultivating at least one cobalt-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is cobalt;
20 (c) harvesting said at least one plant; and
(d) recovering cobalt from said harvested plant.

20. The method of claim 19, wherein the soil has a calcium concentration of between about 20-80%.

21. The method of claim 20, wherein said at least one plant is an *Alyssum* plant.

22. The method of claim 21, wherein said *Alyssum* plant is selected from the group consisting of: *A. murale*, *A. pintodasilvae*, *A. serpyllifolium*, *A. malacitanum*, *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A. bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum*, *A. caricum* and mixtures thereof.

23. An isolated *Alyssum* plant species that accumulates nickel in quantities greater than that accumulated by *A. murale* 103.

24. An isolated *Alyssum* plant species cultivated on nickel-containing soil that accumulates nickel in above-ground tissue at a concentration of 1.55% or greater by weight based on the gross dry weight of said tissue.

25. The isolated *Alyssum* plant species of claim 23 or 24, wherein the species is *A. corsicum* G16.

26. The isolated *Alyssum* plant species of claim 23 or 24, wherein the species is *A. murale* G69.

27. The isolated *Alyssum* plant species of claim 23 or 24, wherein the species is *A. murale* G82.

28. Pollen of the plant of claim 23 or 24.

29. A plant having all the physiological and morphological characteristics of the plant of claim 23 or 24.

30. Propagation material of the plant of claim 23 or 24.
31. An *Alyssum corsicum* G16 seed as deposited with the American Type Culture Collection under accession number 203436.
- 5 32. An *Alyssum murale* G69 seed as deposited with the American Type Culture Collection under accession number 203437.
33. An *Alyssum murale* G82 seed as deposited with the American Type Culture Collection under accession number 203438.
34. A plant produced by growing the seed of any one of claims 31-33.
35. Pollen of the plant of claim 34.
- 10 36. A plant having all the physiological and morphological characteristics of the plant of claim 34.
37. Propagation material of the plant of claim 34.
38. A method for decontaminating metal-containing soil, comprising cultivating at least one hyperaccumulator plant in metal-containing soil, whereby the concentration of metal in the above-ground plant tissue of said at least one hyperaccumulator plant exceeds the concentration of metal in said soil by a factor of at least 2.
- 15 39. The method of claim 38, wherein the at least one hyperaccumulator plant exceeds the concentration of metal in said soil by a factor of 3.

40. The method of claim 39, wherein the at least one hyperaccumulator plant exceeds the concentration of metal in said soil by a factor of 4.

41. A method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:

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- (a) lowering the pH of the soil; and
- (b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate said at least one metal from the soil in above-ground tissue.

42. The method of claim 41, wherein said at least one metal is selected from the group consisting of: cobalt, palladium, rhodium, ruthenium, platinum, iridium, osmium, rhenium and mixtures thereof.

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43. The method of claim 41, wherein the pH of the soil is lowered by adding to the soil at least one agent that results in a decrease of the soil pH.

44. The method of claim 41, further comprising after said cultivating:

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- (c) raising the pH of the soil; and
- (d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.

45. The method of claim 44, wherein the pH of the soil is elevated by adding to the soil at least one agent that results in an increase in the soil pH.

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46. The method of claim 45, wherein the at least one agent that results in an increase in the soil pH is selected from the group consisting of limestone, dolomitic limestone, lime, hydrated lime, limestone equivalents and mixtures thereof.

47. The method of claim 44, wherein the at least one second metal is nickel.

Applicant's or agent's file reference number 1797.009PC05	International application No. 05/017087-1 be assigned
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**INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL**
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>11</u> , line <u>2</u> .	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution American Type Culture Collection (ATCC)	
Address of depositary institution (including postal code and country) 10801 University Boulevard Manassas, Virginia 20110-2209 United States of America	
Date of deposit November 6, 1998	Accession Number ATTC 203436
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Alyssum corsicum G16	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Applicant's or agent's file reference number 1797.009PC05	International application No. <u>be assigned</u>
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Name of depositary institution American Type Culture Collection (ATCC)	
Address of depositary institution (including postal code and country) 10801 University Boulevard Manassas, Virginia 20110-2209 United States of America	
Date of deposit <u>November 6, 1998</u>	Accession Number <u>ATTC 203437</u>
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
This information is continued on an additional sheet <input type="checkbox"/>	
<u>Alyssum murale G69</u>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

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Applicant's or agent's file reference number 1797.009PC05	International application No. 1797.009 be assigned
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**INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL**
(PCT Rule 13bis)

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B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection (ATCC)	
Address of depositary institution (including postal code and country) 10801 University Boulevard Manassas, Virginia 20110-2209 United States of America	
Date of deposit November 6, 1998	Accession Number ATTC 203438
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Alyssum murale G82	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/26443

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C21B 9/00; C22B 9/00; A01H 3/02, 5/00; A01G 1/00

US CL :75/710; 800/276, 260; 210/602, 681, 682

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 75/710; 800/276, 260; 210/602, 681, 682

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS, DIALOG, WEST1.2a

TERMS: PHYTOEXTRACTION, PHYTOMINING, PHYTOREDUCTION, NICKEL, COBALT, HYPERACCUMULATORS, ALYSSUM, SOIL PH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 5,711,784 A (CHANEY et al) 27 JANUARY 1998, see whole document.	1-4, 8-14, 19-22, 29-30 ----- 15-18, 23-40
X - Y	US 5,785,735 A (RASKIN et al.) 28 JULY 1998, see columns 6-7.	19, 41-43, ----- 20-22,
Y	RASKIN et al. Bioconcentration of Heavy Metals by Plants. Current Opinion in Biotechnology. 1994, Vol. 5, pages 285-290, see pages 287-288.	5-7, 44-47



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

27 JANUARY 2000

Date of mailing of the international search report

09 MAR 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/26443

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROMERO et al. Metal Plant and Soil Pollution Indexes. Water, Air, and Soil Pollution. 1987, Vol. 34, No. 4, pages 347-352, see entire document.	1-4, 8-18, 23-40
Y	KUMAR et al. Phytoextraction: The Use of Plants to Remove Heavy Metals from Soils. Environ. Sci. Technol. 1995, Vol. 29, no. 5, pages 1232-1238, see entire document.	19-22, 41-43
Y,P	ROBINSON et al. Soil Amendments Affecting Nickel and Cobalt Uptake by Berkheya coddii: Potential Use for Phytomining and Phytoremediation. Annals of Botany. 1999, Vol. 84, pages 689-694, see page 691-694.	5-7, 44-47
Y,P	US 5,927,005 A (GARDEA-TORRESDEY et al.) 27 July 1999, see entire document.	1-4, 8-18, 23-40
A,P	US 5,928,406 A (SALT et al.) 27 July 1999, see entire document.	19-22, 41-43
Y,P	US 5,917,117 A (ENSLEY et al.) 29 June 1999, see columns 1-2, 6-8, 13-14.	5-7, 44-47
Y,P	US 5,944,872 A (CHANNEY et al.) 31 August 1999, see entire document.	1-4, 8-18, 23-40

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-4, 8-18, 23-40, drawn to a method for selectively recovering nickel by elevating soil pH.

Group II, claim(s) 19-22, 41-43, drawn to a method for recovering cobalt from contaminated soil by lowering soil pH.

Group III, claim(s) 5-7 and 44-47, drawn to a method for sequentially recovering 2 metals from soil comprising the first step of raising or lowering soil pH, followed by the second step of altering pH in the opposition direction of the first step.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The claimed method for recovering at least one metal from metal contaminated soil by elevating the pH of soil and phytomining is anticipated by each of Chaney et al and Raskin et al, as set forth in the Search Report, and so do not constitute a single special technical feature which would be an advance over the prior art.

The invention of Group I, drawn to a first method for recovering nickel from nickel-contaminating soil, requires presence of in the soil, limestone and limestone equivalents to increase soil pH not required by any other group.

The invention of Group II, drawn to a second method for recovering cobalt from cobalt-contaminated soil, requires presence of cobalt in the soil, cobalt uptake by a plant, and a lowered pH not required by any other group.

The invention of Group III, drawn to a third method for sequentially recovering nickel and cobalt, requires a raised soil pH followed by a lowered soil pH or vice-versa not required by any other group.